

AN AFM-BASED STUDY OF PORCINE OBP FILM FOR ARTIFICIAL OLFACTION

Qiang Cai, Min Pan, Guang Li, Yuquan Chen

Biosensor National Special Lab., Zhejiang University, Hangzhou, Zhejiang, P.R.China

Abstract- The odorant binding protein (OBP) plays an important role in olfactory signal transmit. This paper studied the potential application of OBP on bionic artificial nose. Above all, OBP is immobilized in solid phase. So two kinds of OBP film were prepared. Firstly, OBP is covalently immobilized on the silicon wafer using DITC as a crosslinker. Secondly, polypyrrole film was fabricated on the ITO glass by the electrochemical polymerization. The topograph of these films was measured using Atomic Force Micrograph (AFM). In order to evaluate the affinity of OBP and its antibody, the AFM probe was coated with the anti-OBP using the covalent method. The force-distance curve, acquired by the AFM instrument, was analyzed to reveal the characteristic of thin film and the binding property between OBP and antibody.

Keywords - AFM, Rupture Force, Polypyrrole, OBP

I. INTRODUCTION

Odorant-binding protein (OBP, 36,000Mr) of vertebrates, belonging to a class of soluble proteins, lipocalin family, are expressed in the nasal glands, released into the mucus and are thought to transfer the hydrophobic odorous molecules through the aqueous mucus barrier towards the olfactory sensory neuron [1]. Although their functions have not been known clearly, it is apparent that OBP plays a much more important role in olfaction for their highly concentration in the nasal mucus. There are several theories and models to define the mechanism of releasing and modulating chemical messages via OBPs. From the view of bionics, OBP has the potential application on Artificial Nose with practical possibilities for they are easier to isolate and produce than receptor of odorant in olfactory epithelial cell. The binding properties and specificity shall be investigated to provide the basis of biosensor. In 1996, Mario A. Bianchet and Paolo Pelosi investigated the three-dimensional structure of bovine odorant binding protein and its mechanism of odor recognition measured by electron density maps, which is calculated with those heavy atom derivatives using the Pt anomalous scattering data [2]. In 1999, Paolini S. and Tanfani F. measured structural stability and ligand affinities by Fourier-Transform Infrared-Spectroscopy and Fluorescence Spectroscopy [3]. All those works show the possibility of OBPs as biosensor, however, it is not enough to construct a biosensor.

In order to develop a prototype of gas sensing bionic transducer, four porcine OBP, highly expressed in the nasal area and classified as OBP on the basis of their binding affinity to 2-isobutyl-3-methoxypryrazine, was purified. This paper developed the evaluation method of the binding

properties through the analysis of force-distance curve acquired from AFM, as well as surface characteristics of the OBP film. This work was done to assure the binding activity of immobilized OBP. It is the basis of artificial nose using fluorescence detection, because fluorescence can be detected when odorant are captured, if the immobilized molecules are fluorescence labeled OBP.

II. METHODOLOGY

Porcine OBP was extracted from the pig nasal epithelium. These tissues were dissected from individuals and prepared by homogenization in 20mM Tris/HCL buffer, pH7.4(Tris buffer), using a Polytron apparatus, followed by centrifugation a 10,000 g for 20 min. The clear supernatant was used for electrophoretic analysis and chromatographic fractionation. And then the extract was concentrated to 0.5 ml and separated by gel filtration on Superose 12 column ($1 \times 30\text{cm}$) eluted with 50 mM ammonium bicarbonate, 1.0 ml fractions being collected. After SDS/PAGE (12% gels), tubes containing proteins of interest were pooled and subjected to anion-exchange chromatography through a Mono ! HR 5/5 column. A linear gradient from 0 to 0.5M NaCl in 20 mM Tris/HCl buffer, pH7.4, was used for elution. This procedure yielded a mixture of 14-kDa protein isoforms, which was used for antibody production of further purified by reversed-phase HPLC before structural investigations. Individual components of the family were isolated in homogeneous form by chromatographic step on a Vydac C₄ 214TP54 column ($250 \times 4.6\text{mm}$; $5 \mu\text{m}$; 30nm pore size). Proteins were dissolved in 0.1% trifluoroacetic acid, loaded on to the column and eluted with a linear gradient of from 5 to 70% acetonitrile in 0.1% trifluoroacetic acid over 30 min, at a flow rate of 1 ml/min[4].

To detect the binding properties between OBP and anti-OBP, it is necessary to immobilize them to a solid phase. The two approaches are applied for immobilization. One is covalent binding, the other is polymerization.

In covalent binding methods, n-Type silicon wafers were adopted as substrates. First of all, these substrates should be treated carefully because any contamination would lead to failure. So these silicon chips were washed several times using 37%HCl, de-ionized water and methanol. To immobilize the OBP or its antibody, those chips should be preprocessd. Firstly, they were oxidized by immersing into 70%H₂SO₄, 15% H₂O₂, 15% H₂O at 60°C for one hour. The surfaces were subsequently rinsed several times in respectively de-ionized water and methanol. While dried, they reacted in 5% H₂N(CH₂)₃Si(OCH₃)₃ solution in toluene at 60°C for half an hour. Then they were immersed into 2% DITC (1,4-phenylene diisothio-cyanate) in toluene at 60°C

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for half an hour, after they were washed several times by toluene and methanol. These steps prepared the stable immobilizing film for Porcine OBP or its antibody. Also the AFM tip was processed using the same method.

Porcine OBP solution (0.1 μ g/ml Porcine OBP in the NaHCO_3 solution) was dipped on those processed chips or AFM tips and incubated at 50°C for ten minutes. It is similar to immobilized the antibody of porcine OBP. In order to maintain the bioactivity of these films, they were stored in 0.01 mol/L NaHCO_3 in de-ionized water at 4°C.

The other immobilization, depending on the deposition of polypyrrole on the ITO glass, shows the ability to control the immobilizing film. The more conducting the glass is, the better is the formed polypyrrole film. The attachment of macromolecular is based on electrostatic force. Different polymerization method to form this film has been tried, and the electripolymerisation displays better characteristics than the others. The electrolyte solution was prepared before electropolymerization. The solution contained 0.1M pyrrole monomer, 0.1M $\text{Et}_4\text{NH}_4\text{BF}_4$ and 1% H_2O in acetonitrile. The deposition of polypyrrole film were carried out on an EG&G P.A.R. Model 273A bipotentiostat (USA). The cell (See Fig. 1) was fabricated and applied to deposit the film by three-electrode mode. The different voltages and duration wielded in the electropolymerization led to different disposition rate, thickness and surface roughness.

In order to evaluate binding properties of sensing film, the topography was measured in advance to select the smoother film. Using 'touch and lift' scanning mode [5], AFM was used to detect rupture force between OBP and its antibody (See Fig. 2). The binding properties were revealed by those acquired force-distance curves. All these measurements were executed in NaHCO_3 solution (pH is equal to 8.3) to remove the meniscus force. In addition, the AFM instrument was calibrated by 25.5 nm and 105 nm silicon grating before measurement.

The topography data were processed using SPIP 1.51, issued by Image Metrology ApS. The force-distance curves were analyzed using Origin 5.0, issued by Microcal Software Inc.

III. RESULTS AND DISCUSSION

According to the above protocol, the topograph of prepared films using covalent method and polymerization are shown in Fig. 3. Fig. 3 (a) and (b) indicate the height span 100nm. In Fig. 3 (c) and (d), the dark side is the polypyrrole film and the white side is the ITO glass. It shows the thickness of the film is about 200nm. These films are smooth enough to detect rupture force. Although the polypyrrole films are much smoother than those prepared by covalent method, it does not mean that polymer film is much better than covalently modified film. Because the covalent binding force is much stronger than the electrostatic force, the covalently modified film is more stable. The attached force between polymer film and substrate is often so weak that it is possible to pull them away from the substrate by the Antibody modified AFM tip. The rupture force curves are shown in Fig. 4 and Fig. 5. The main 'jump' from "contact"

to "off" is equal to 1000pN and 3000pN. There are some small 'jumps' apparently shown in Fig. 5. This kind of rupture force is about 10~100pN. It is ascribed to different binding sites holding different binding force. The binding site locating outside of the cup structure of OBP shows the relatively weak binding force. So it proves existence of auxiliary binding sites.

The bioaffinity can be evaluated based on these force-distance curves. Because the bioaffinity depends on the amount and orientation of immobilized biomolecules. The amount of the rupture force also depends on the amount and orientation of immobilized biomolecules. Therefore, the more rupture force is, the higher loading of biomolecules is indicated. Because the same modified AFM tip was wielded to detect the rupture force of two films, the difference of those force-distance curves indicated the amount or orientation of force-distance. Comparing the Fig. 4 and Fig. 5, the main rupture force is about 6000pN in Fig. 5, and 1000pN in Fig 4. And the two main "jump" were found in Fig 5. That means, according to the above theory, there were much more biomolecules (POBP) immobilized on the solid phase.

When we use AFM tip without any modification to detect those POBP modified films, we have not found the apparent rupture force. On the other hand, the anti-OBP modified AFM tips are used to detect polypyrrole film, there is also not apparent rupture force too. Therefore, It proves that the 'jump' (displayed in Fig 4 and Fig. 5) due to the antigen and antibody binding reaction.

IV. CONCLUSION

The OBP has enough strong binding force and many binding sites. And it is efficient to immobilize the OBP by means of covalent method or polymerization. Because dissociation between OBP and odorant is also possible, the potential application of OBP as biosensor is implied. Because the odorant is so small considering OBP, it is not easy to detect the binding between OBP and odorant by means of dielectric constant or mass. The possible resolution is to label the OBP and detect the fluorescence spectra. The fluorescent photoaffinity labelling porcine OBP and its detection have been reported in some research [6]. So it is possible that the above OBP film can be used as artificial nose based fluorescence detection.

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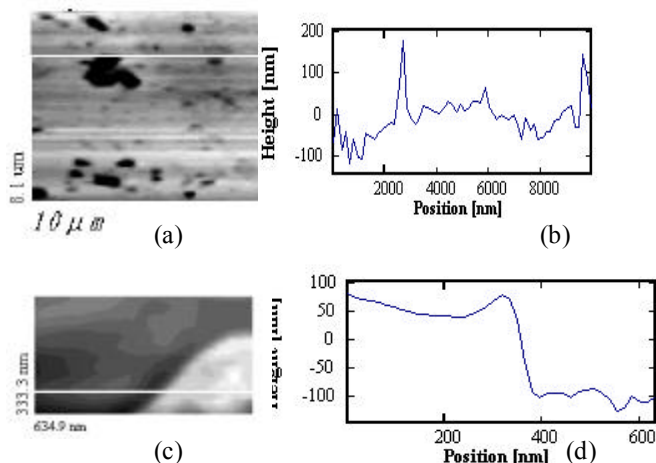
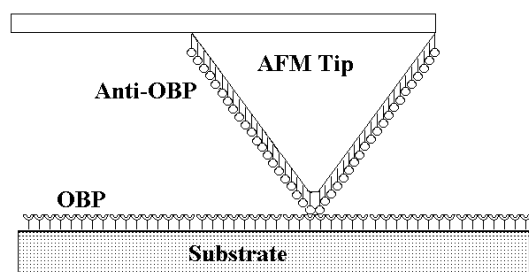
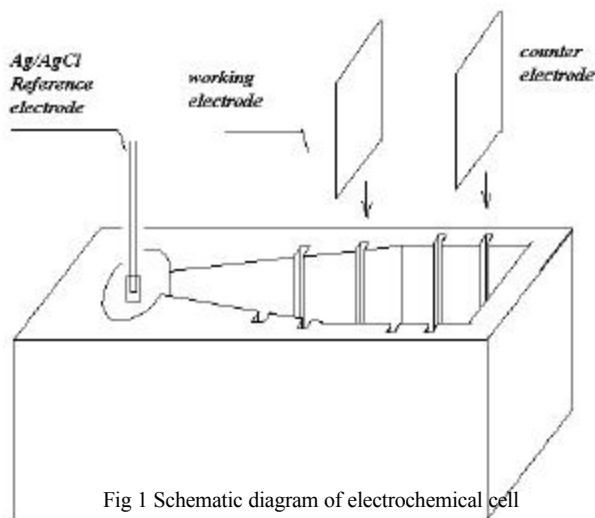


Fig 3 the film prepared by covalent method

(a) the topograph of film prepared by covalent method

(b) the height line indicating the white line in the topographn (a)

(c) the topograph of film prepared by polymerization method

(d) the height line indicating the white line in the topographn (c)

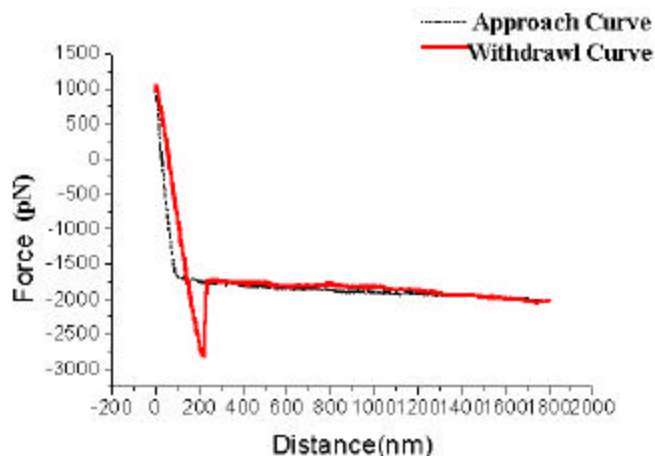


Fig 4 Force-Distance Curve on the covalent film

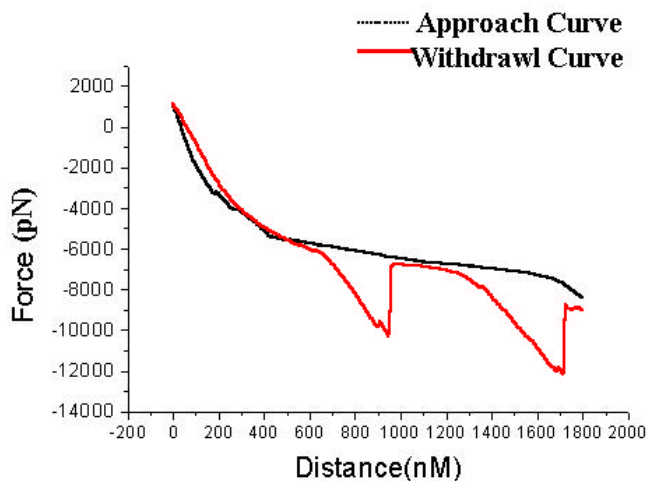


Fig 5 Force-Distance Curve on the polymer film